IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Gregory A. Demopulos et al.	Attorney Docket No:	CH.1.0018.US2
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Filed:	January 18, 2002	Examiner:	Micah Paul Young

Title: SOLUTIONS AND METHODS FOR INHIBITION OF PAIN, INFLAMMATION AND CARTILAGE DEGRADATION

DECLARATION UNDER 37 CFR §1.132

TO THE ASSISTANT COMMISSIONER FOR PATENTS:

I, Emma Elizabeth (Betsy) Moore, Ph.D., hereby state:

1. I hold a doctorate in Biophysics and Genetics from the University of Colorado Health Sciences Center in Denver, Colorado, and completed postdoctoral fellowships at the Centre de Genetique Moleculaire in France and the Eleanor Roosevelt Institute for Cancer Research in Denver, Colorado. Following my postdoctoral work, I worked as an Assistant Professor at the University of Colorado Health Sciences Center for five years, and then a Staff Scientist at the National Jewish Hospital and Research Center, Denver, Colorado, for two years. For the last ten years, I have worked in industry as a scientist and have spent much of the past six years conducting research into chondrogenesis and cartilage repair. I am currently a Senior Group Leader at Omeros Corporation in Seattle, Washington, where I lead a team of scientists developing chondroprotective products.

2. I have reviewed the above-identified patent application, which claims methods for inhibiting cartilage degradation by locally delivering to a joint a composition including at least one anabolic chondroprotective agent and at least one inhibitor of

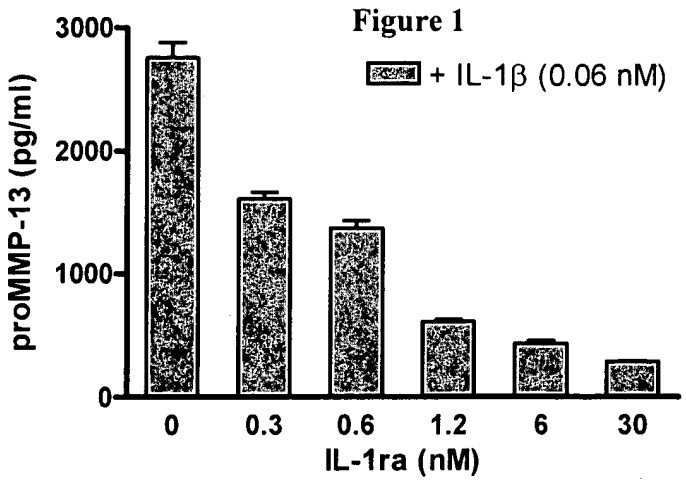
cartilage catabolism. I am aware that an Office Action was generated in the above-identified application on May 4, 2006, which rejected the pending claims of the application as being unpatentably obvious, and invited the Applicants to submit experimental data in support of the claimed methods.

3. I personally directed a series of experiments evaluating inhibitors of cartilage catabolism and anabolic chondroprotective agents, as single agents and in combination, in established models of osteoarthritis using standard assays measuring markers of cartilage catabolism or cartilage synthesis. Representative reproduced (except as noted) data resulting from these experiments and experimental methods used are summarized below as Examples 1 through 4.

Example 1: The combination of IGF-1 + IL-1Ra is more effective than each agent alone in restoring matrix homeostasis to IL-1 treated chondrocytes

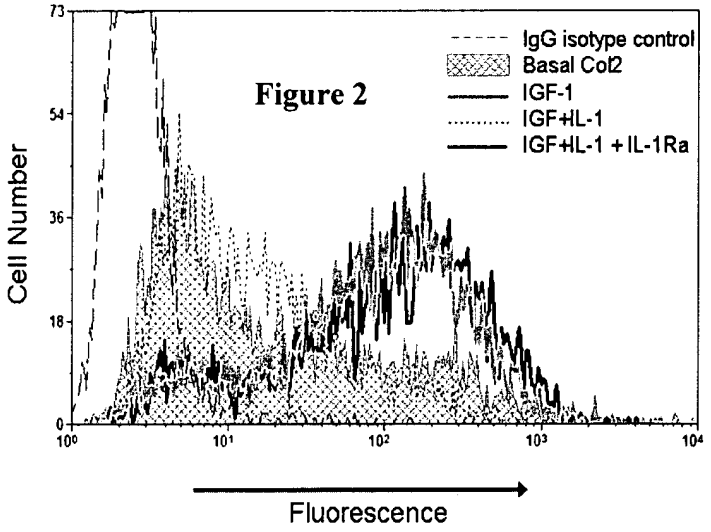
4. IL-1 is a well-established catabolic agent whose expression is elevated in the osteoarthritic joint [1]. It is well documented that IL-1 will induce chondrocyte expression of proteolytic enzymes such as matrix metalloproteases (MMPs) and aggrecanases (ADAMTS) that degrade the two major components of the cartilage matrix, type II collagen (Col2) and aggrecan [2;3]. The catabolic inhibitor IL-1Ra (IL-1 receptor antagonist) specifically blocks the activity of IL-1β and its induction of matrix degradation [4].

5. MMP-13 is thought to be one of the major proteases involved in the degradation of type II collagen in the osteoarthritic joint so that any agent that blocks its production and/or activity should have a beneficial effect in the osteoarthritic joint [3]. As shown in Figure 1, IL-1 will induce the expression of proMMP-13, the precursor form of active MMP-13, in human immortalized chondrocytes (C28/I2 cells) and IL-1Ra will inhibit this induction in a concentration-dependent manner. Experimental details for this example are described below in the Methods. The half-maximal inhibitory concentration for IL-1Ra was 0.6 nM or ten-fold higher than the exposure concentration of IL-1β (0.06



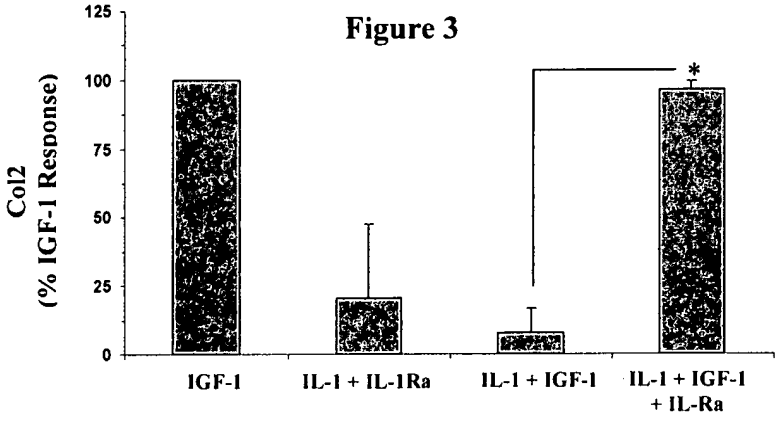
nM). A concentration of 30 nM IL-1Ra completely blocked IL-1β stimulatory activity, returning proMMP-13 production to basal levels (data not shown).

6. Although IL-1Ra can effectively block IL-1 induction of matrix degradation, it cannot by itself stimulate synthesis of new matrix to replace lost material. Furthermore, IGF-1, a well-established anabolic agent for cartilage, cannot induce matrix synthesis in the face of an IL-1 challenge. However, treating the IL-1 stimulated chondrocytes with the combination of IL-1Ra and IGF-1 restored the ability of the chondrocytes to respond to IGF-1 and synthesize increased amounts of the two major matrix components, type II collagen (Col2) and aggrecan. These results are illustrated in Figures 2-4 and the experimental details are described in Methods. **Figure 2** shows the effects of various 48-hour treatments on the production of intracellular Col2 by primary bovine chondrocytes (BACs) as measured by Fluorescent-Activated Cell Sorting (FACS) analysis. Col2 positive cells were identified as those that had a fluorescent intensity greater than the IgG



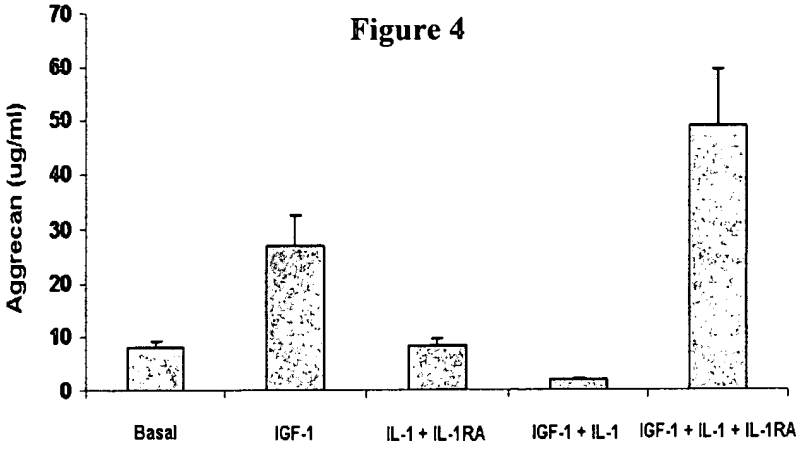
negative isotype control (> 10). Each treatment group was evaluated by their percentage of Col2 positive cells and their mean fluorescent intensity (MFI). In this particular study, IGF-1 treatment increased the percentage of cells expressing Col2 (Col2 positive) from the unstimulated level of 60% to 90% and increased the amount of MFI from 113 to 168. However, the addition of IL-1β completely blocked the effects of IGF-1 so that there was no significant increase in the percentage of Col2 positive cells over untreated levels (63% vs 60%) and a decrease in the MFI (42 vs. 113). The addition of the catabolic inhibitor, IL-1Ra, was able to prevent the loss of the anabolic effects of IGF-1 in the presence of IL-1, resulting in 90% Col2 positive cells with a MFI of 206.

7. **Figure 3** shows quantitative FACS results obtained from three independent cultures of primary bovine chondrocytes (SEM, n=3). All data was normalized to the maximal IGF-1 response which is defined as the difference in the percentage of Col2-positive cells in the IGF-1 treated vs. untreated control cultures. IL-1β decreased this IGF-1 response by 90%; however, this inhibition was blocked by a 100-fold molar excess of IL-1Ra (* $p < 0.001$, unpaired Student's T-test). The addition of IL-1Ra to the IL-1β stimulated BAC cultures resulted in a slight, but non-significant increase in the percentage of Col2 positive cells and this increase is clearly much smaller than that induced by the combination of IGF-1 + IL-1Ra.



8. Aggrecan is the major proteoglycan found in the cartilage matrix and its synthesis is induced by IGF-1 [5]. However, similar to the results obtained for Col2, the IGF-1 induction of aggrecan synthesis is inhibited by IL-1β and the addition of IL-1Ra will reverse this inhibition. The amount of aggrecan chondroitin sulfate released into the culture medium of primary BAC was monitored with a specific immunoassay that detects newly synthesized aggrecan (CS-846 ELISA) as described in the Methods below and the data is shown in **Figure 4**. Aggrecan synthesis was stimulated three-fold in response to 48-hour treatment with IGF-1 (50 ng/mL). The addition of IL-1β (10 ng/mL) reduced aggrecan synthesis to levels that were even lower than those found in the untreated control cultures. Treatment of IL-1β stimulated BAC cultures with the combination of IGF-1 and IL-1Ra not only resulted in full restoration of the anabolic effect of IGF-1 but the amount of aggrecan was five-fold greater than that seen in the basal cultures and almost 1.5 to two-fold greater than the cultures treated with IGF-1 in the absence of IL-1.

Figure 4



9. In addition to IL-1Ra, a variety of other catabolic inhibitors were evaluated for their ability to a) inhibit the IL-1 induction of pro-MMP-13 or b) reverse the IL-1 inhibition of IGF-1 induction of Col2 and/or aggrecan. Several signal transduction inhibitors were identified that suppressed the IL-1 induction of pro-MMP13 including inhibitors of the ERK1/2, JNK, and p38 MAPK pathways as well as an inhibitor of the NF-κB pathway (data not shown). However, none of these inhibitors

reversed the IL-1 inhibition of IGF-1 induction of Col2 or aggrecan under the experimental conditions that were used (data not shown).

10. The results presented in **Example 1** clearly demonstrate that a combination of a catabolic inhibitor, IL-1Ra, and an anabolic factor, IGF-1, would be expected to have a significantly greater chondroprotective effect when delivered locally to an osteoarthritic joint than either agent alone. IL-1Ra would inhibit the degradation of cartilage matrix that is mediated by IL-1 induced proteases (e.g. MMP-13); however, it would have little effect on the regeneration of cartilage matrix. IGF-1 would have minimal effect on the degradation of type II collagen, and it would not be able to stimulate the synthesis of cartilage matrix in the presence of elevated levels of IL-1β that are found in the osteoarthritic joint. However, local administration of IGF-1 + IL-1Ra is predicted to inhibit cartilage degradation and restore the anabolic effects of IGF-1 thereby leading to repair of damaged matrix.

Methods for Example 1

11. **Immortalized Human Chondrocyte Cells:** The immortalized human chondrocyte cell line, C28/I2, was obtained from Mary B. Goldring, Ph.D. and maintained as previously described [6]. The growth medium consisted of DMEM/F12 with GlutaMax™ (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 50 units/mL of penicillin/streptomycin (Invitrogen, Carlsbad, CA). The basal medium consisted of DMEM/F12 with GlutaMax™ supplemented with 500 ng/mL fatty acid-free bovine serum albumin, 50 μg/mL human transferrin, 5 ng/mL selenium and 50 units/mL of penicillin/streptomycin. Unless indicated, all additives were purchased from Sigma-Aldrich.

12. **Collagenase Production in Immortalized Human Chondrocytes:** C28/I2 cells were seeded into multi-well tissue culture plates at an initial density of 25,000 cells per cm^2 and grown to 80% confluence. The growth medium was replaced with serum-free basal medium and the cultures allowed to incubate an additional 24

hours. Following equilibration, the basal medium was replaced with fresh basal medium and the cells received a 20 minute pretreatment with increasing concentrations of IL-1Ra prior to the addition of 1 ng/mL IL-1β (60 pM). Twenty-four hours later the culture medium was collected, centrifuged at 10,000 rpm and analyzed for proMMP-13 (collagenase-3) content utilizing a commercially available human proMMP-13 ELISA kit (R & D Systems). Quantization of proMMP-13 content was derived from standard curve profiles and expressed as pg/mL levels.

13. **Primary Bovine Articular Chondrocyte (BAC) Isolation:** Primary cultures of bovine articular chondrocytes (BAC) were established from the metacarpophalangeal joints of 18- to 24- month-old animals. Cartilage fragments were digested overnight at 37^0 C in 1mg/mL collagenase (Type II, Worthington) prepared in basal medium. Basal medium consists of DMEM/F12 (Gibco) supplemented with GlutaMax™ (Invitrogen, Carlsbad, CA) , 1 mM pyruvate, 25 mM Hepes, 500 ng/mL fatty acid-free bovine serum albumin (BSA), 50 units/mL of both penicillin/streptomycin, and 500 ng/mL Fungizone (Invitrogen, Carlsbad, CA). The dissociated cells were washed three times in PBS, resuspended in serum-free defined medium and seeded into multi-well culture plates at a density of 500,000 cells per cm^2. Serum-free defined medium consists of basal medium supplemented with 50 μg/mL human transferrin, 5 ng/mL selenium, and 50 μg/mL freshly prepared ascorbic acid. All medium additives were purchased from Sigma-Aldrich unless indicated otherwise. Cultures were equilibrated prior to assay by incubating for 72 hours at 37°C in 5% CO_2 atmosphere.

14. **Anabolic Response in BAC:** All treatment conditions were performed in serum-free defined culture medium. Two sets of culture conditions were compared. One set of chondrocyte cultures was preincubated for 20 minutes at 37°C with 1 μg/mL (60 nM) of recombinant, human IL-1Ra (R&D Systems) prior to receiving cytokine additions. This preincubation allows for IL-1Ra to bind to the type I IL-1 receptor and interfere with subsequent IL-1 ligand interaction. By comparison, a second set did not

receive IL-1ra pretreatment. Both sets of cultures received subsequent additions of either 50 ng/mL recombinant human IGF-1 (R&D Systems), 10 ng/mL (600 pM) recombinant bovine IL-1β (SeroTec) or the combination of IGF-1 + IL-1β. Untreated cultures were included in both treatment sets to serve as basal level controls. Cultures were incubated for 48 hours and then monitored for two independent markers of anabolic activity: the synthesis of collagen type II (Col2) and the synthesis of aggrecan proteoglycan.

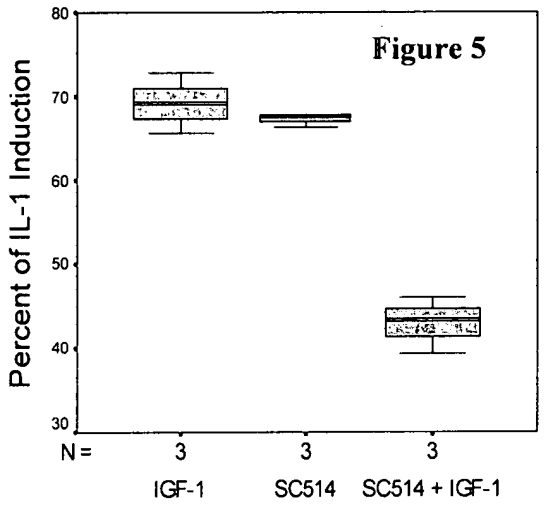
15. **Synthesis of Collagen Type II in BAC:** Intracellular levels of Col2 were evaluated by FACS analysis according to the protocols established by Verbruggen *et al.* [5]. Following experimental treatments, BAC monolayer cultures were digested with collagenase (Worthington Type 2, 1 mg/mL) for 1 hour at 37°C to obtain a single cell suspension and to remove the cell-associated matrix. Cells were washed three times in PBS, fixed in 5% neutral buffered formalin for 30 minutes at room temperature and then permeabilized in ice cold 90% methanol for 10 minutes. Fixed and permeabilized cells were then resuspended in blocking buffer: PBS containing 0.5% fetal bovine serum and 100 μg/mL human IgG (Sigma-Aldrich) and refrigerated overnight. Cell suspensions were incubated for 30 minutes at room temperature in blocking buffer containing primary 1 μg/mL anti-collagen type II monoclonal antibody, AB-1 (clone 11-4C11, Calbiochem). Cells were washed extensively and resuspended in PBS/FBS containing 1 μg/mL of AlexaFluor 488 conjugated goat anti-mouse IgG antibody (Molecular Probes/Invitrogen). Samples were washed extensively in PBS/FBS, kept dark on ice and immediately analyzed for immunofluorescence using a FACS Caliber flow cytometer. Control staining consisted of cells incubated with conjugate alone, allowing for proper instrument adjustment of background fluorescence. The data were analyzed and plotted using Summit® software from Cytomation. Positive fluorescence accurately represents intracellular Col2 levels since no cell surface fluorescence was detected following collagenase digestion (data not shown).

16. **Synthesis of Aggrecan Proteoglycan in BAC:** Newly synthesized aggrecan is secreted from chondrocytes as a large aggregating proteoglycan containing the chondroitin sulfate moiety CS-846. Culture medium collected from the cell monolayers utilized to determine Col2 synthesis above was analyzed for newly synthesized aggrecan content utilizing a CS-846 specific ELISA (IBEX). The ELISA procedure was performed according to manufacturer's recommendations and the amount of CS-846 epitope was quantified from internal standards. Aggrecan content of culture medium is expressed as μg/mL.

Example 2: The combination of IGF-1 and SC514 or UO126 is more effective than each of the respective agents at blocking IL-1 induction of aggrecanase activity.

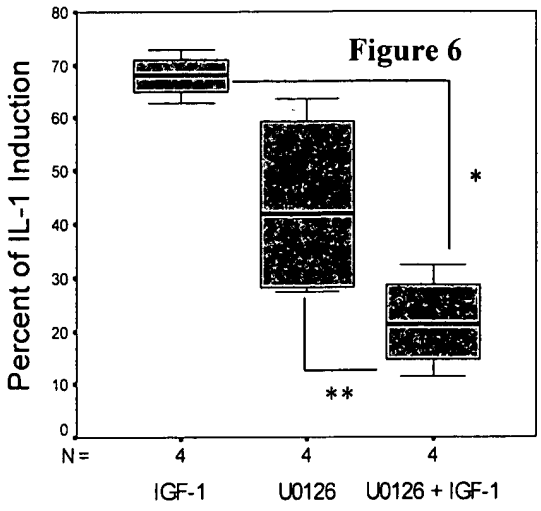
17. The destruction of aggrecan in the osteoarthritic joint is attributed to increased activity of aggrecanase (ADAMTS-4 and -5) which is induced by IL-1 and other cytokines [2]. Hence, any agent that inhibits either the production and/or activity of aggrecanase would be expected to exert a chondroprotective effect in an osteoarthritic joint. Control primary cultures of bovine chondrocytes (BACs) express undetectable levels of aggrecanase activity and treatment with IL-1β (10 ng/mL) routinely induced a 20-fold increase in this activity. Preliminary studies were performed to evaluate the effect of four different catabolic inhibitors, either alone or in combination with IGF-1, for their ability to inhibit the IL-1 induction of aggrecanase activity. These catabolic inhibitors included a) a p38 MAPK inhibitor, SB239063, b) an Extracellular-signaling Regulated Kinase 1/2 (ERK1/2) MAPK inhibitor, U0126, c) a c-Jun N-terminal Kinase (JNK) MAPK inhibitor, SP600125, and d) a NF-κB inhibitor, SC514. Three different concentrations of each agent were tested either alone or in combination. The results with the JNK inhibitor were highly variable, whereas the p38 inhibitor by itself effectively blocked the IL-1 induction of aggrecanase activity at the concentrations evaluated.

Therefore, only the combinations of IGF-1 + SC514 or IGF-1 + U0126 were subjected to additional analysis and these data are presented in **Figures 5 & 6**.



18. **Figure 5** shows the results from three independent studies with IGF-1 and the NF-κB inhibitor, SC514. The details of the aggrecanase activity assay are described in the Methods below and the data is presented as the percent of IL-1 induced aggrecanase activity. The bar corresponds to the median value, the boxes above and below the bar indicate the range of the interquartile values, and the lines indicate the range of all data used for analysis. IGF-1 (50 ng/mL) partially inhibits the IL-1 induction of aggrecanase activity in BACs to 69% +/- 2% (SEM) of the maximal response. SC514 is a potent and selective inhibitor of IKK-2 and suppresses the activation of the NF-κB signal transduction pathway. SC514 (20 μM) acts as an anti-catabolic agent by suppressing the IL-1 induction of PGE_2 in primary bovine chondrocytes to <3% of the IL-1 induced levels (Data not shown). However, in the same cultures, SC514 (20 μM) only partially suppressed the IL-1 induction of aggrecanase to 69% +/- 1% (SEM) of the maximal response. Moreover, the combination of IGF-1 (50 ng/mL) and SC514 (20 μM) inhibited the IL-1 induced aggrecanase activity to 43% +/- 1% (SEM) of the maximal response, thus achieving substantially greater inhibition than either agent alone ($p < 0.001$, LSD ANOVA analysis). It was also surprising to find that IGF-1 had an effect on the aggrecanase pathway, exhibiting an anti-catabolic effect on aggrecanase activity.

19. Similar data was obtained for IGF-1 and a second catabolic inhibitor, U0126, which inhibits the activation of the ERK1/2 MAP kinase signal transduction pathway. The anti-catabolic effect of U0126 (10 μM) is apparent by its ability to inhibit the IL-1 induction of PGE_2 in primary bovine chondrocytes to <1% of induced levels (Data not shown). However, in these same cultures, 10 μM U0126 only achieved partial inhibition of IL-1 induced aggrecanase activity to 43% +/- 10 % (SEM) of the maximal response. The data shown in **Figure 6** was compiled from four independent experiments and demonstrates that the combination of IGF-1 and 10 μM U0126 inhibited IL-1-induced aggrecanase activity to 20% +/- 5 % (SEM) of the maximal response thus achieving greater inhibition of IL-1-induced aggrecanase activity than either agent alone (*p<0.001, **p<0.05, LSD ANOVA analysis). The data is depicted as described for Figure 5.



20. In summary, the data presented in **Example 2** demonstrates that for the concentrations of agents used, the combination of an anabolic agent (IGF-1) and one of either two anti-catabolic agents (U0126 or SC514) is more effective than each of the respective single agents in suppressing the IL-1 induction of aggrecanase activity. By extension, treatment of an osteoarthritic joint by local delivery of a combination of IGF-1 + SC514 or IGF-1 + UO126 should be more effective at inhibiting the degradation of aggrecan than treatment with any of these agents alone and thus provide greater benefit to the patient than using only one of these agents alone.

Methods for Example 2

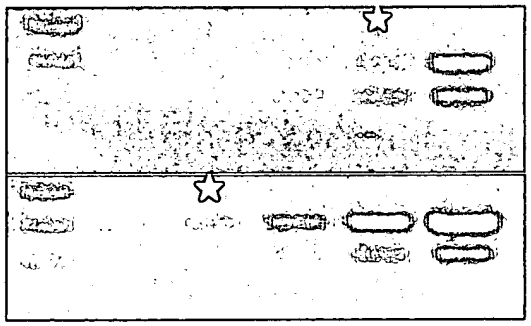
21. ***In Vitro* Analysis of Aggrecanase Activity:** Primary bovine chondrocyte cultures were prepared as described in Example 1. Cells were plated in 48-well plates at 5 x 10^5 cells per well and equilibrated for 72 hours before treatment. The catabolic inhibitors, U0126 and SC514, or appropriate DMSO controls were added 30 minutes prior to addition of IL-1 and IGF-1, and treatment continued for 96 hours. At the end of the treatment period, culture media was collected, frozen at -20° C, and subsequently analyzed for PGE_2 content as described below. The aggrecanase activity associated with the cell monolayers was immediately measured using a modification of a commercial sensitive aggrecanase activity assay kit (MD Biosciences). This commercial kit provides an artificial aggrecanase substrate that corresponds to the interglobulin domain of aggrecan where aggrecanase cleavage occurs and an ELISA kit to quantify the specific aggrecan neoepitope (ARGSVIL) that is generated by aggrecanase cleavage. A reaction mixture containing the artificial aggrecanase substrate was prepared according to the manufacturer's protocol and was incubated with the cell monolayers for 30 minutes at 37°C. The reaction was stopped using the recommended EDTA solution and samples were stored in polypropylene tubes at -20°C until the time of the ELISA assay. An ADAMTS-4 positive control was run in parallel as outlined in the kit protocol. The ELISA was performed according to the standard protocol and the aggrecanase activity calculated from a standard curve of ARGSVIL peptide. Statistical analysis was performed using the SPSS software package (SPSS Inc. Chicago). Differences between the groups were anaylzed using the one-way ANOVA with LSD (Least Significant Difference) Post Hoc test.

22. **PGE_2 Assay:** The PGE_2 content of the media samples was determined using a commercial EIA kit (Cayman Chemical) according to the recommended protocol.

Example 3: The combination of IGF-1 and SC514 or SB239063 is more effective than each of the respective agents at blocking IL-1β induction of MMP-1 mRNA.

23. MMP-1 is a protease that is produced by IL-1 activated chondrocytes and participates in the degradation of type II collagen and aggrecan [3]. Therefore, any agent that inhibits the production and/or activity of MMP-1 would be anticipated to inhibit degradation of cartilage matrix in an osteoarthritic joint. We used RT-PCR to monitor the effects of IGF-1 and four catabolic inhibitors, either alone or in combination, on the IL-1 induction of MMP-1 mRNA in primary BACs. These catabolic inhibitors included a) a p38 MAPK inhibitor, SB239063, b) an ERK1/2 MAPK inhibitor, U0126, c) a JNK MAPK inhibitor, SP600125, and d) a NF-κB inhibitor, SC514. In order to generate semi-quantitative results, three-fold serial dilutions of the cDNA were utilized for each treatment group. Comparison of the level of gene expression between treatment groups was achieved by determining which dilutions generated PCR bands with similar intensity. **Figure 7** illustrates this method of quantitation for the IL-1β induction of MMP-1 mRNA in primary BACs. The left lane contains control MW markers and the next five lanes contain the MMP-1 PCR bands generated by serial dilutions of input cDNA. The dilutions that generated PCR products of equal intensity in the two treatment groups are marked by stars, and comparison of the corresponding dilution factors indicates that IL-

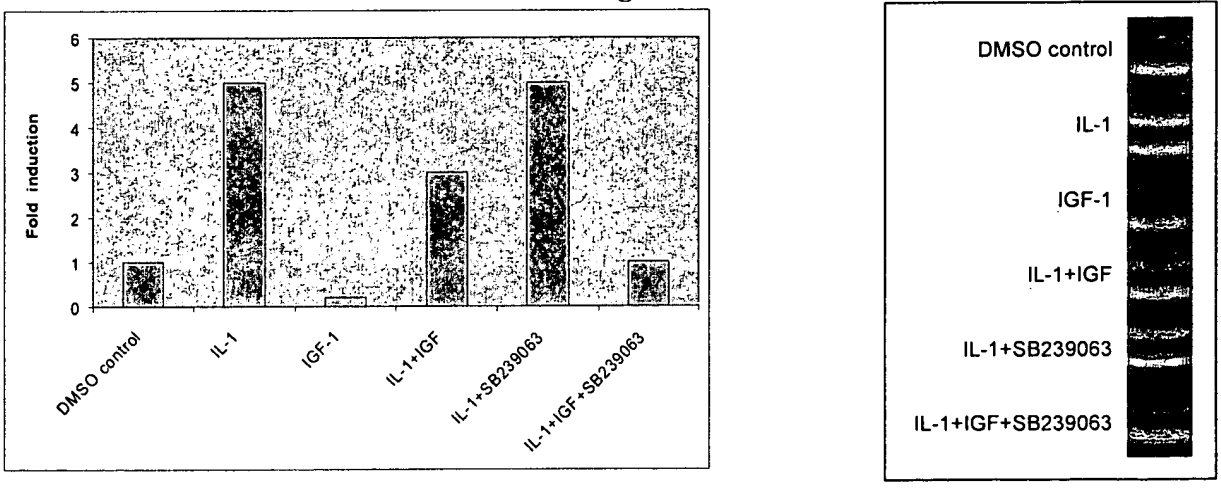
Input cDNA (ng): **0.06 0.18 0.55 1.67 5** **Figure 7**



1β (10 ng/mL) induced approximately a ten-fold increase in the level of MMP-1 mRNA in this experiment.

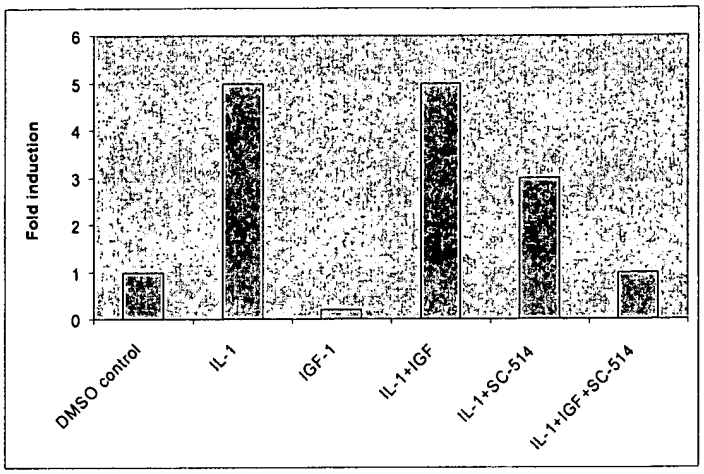
24. Using this approach, we found that two of the four catabolic inhibitors were able to inhibit the IL-1β induction of MMP-1; however, complete inhibition was only achieved in the presence of IGF-1. The experiment was repeated with these two inhibitors and similar results were obtained. The results for IGF-1 (50 ng/mL) and the p38 MAPK inhibitor, SB239063 (10 μM) are shown in **Figure 8.** The panel on the right displays the actual PCR results with equivalent loading of the 18S RNA (lower band) for each treatment group. The upper band in each series corresponds to MMP-1 mRNA, and it is evident that neither IGF-1 nor SB239063 alone significantly inhibited the IL-1 induction of MMP-1 mRNA. However, the combination of IGF-1 and SB239063 completely blocked the IL-1 induction of MMP-1 mRNA. This conclusion is confirmed by the quantitative results from the cDNA dilution series which are shown in panel on the left.

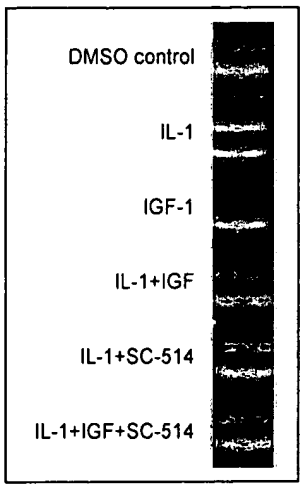
Figure 8



25. Similar results were obtained for the combination of a NF-κB inhibitor, SC514, and IGF-1 and these are shown in **Figure 9**.

Figure 9





26. In summary, the data presented in **Example 3** demonstrates that for the concentrations of agents used, the combination of an anabolic agent (IGF-1) and one of either of two anti-catabolic agents (SB239063 or SC514) is more effective than each of the respective single agents in suppressing the IL-1 induction of MMP-1 mRNA. By extension, treatment of an osteoarthritic joint by local delivery of a combination of IGF-1 + SC514 or IGF-1 + SB239063 should be more effective at inhibiting matrix degradation than treatment with each agent alone.

Methods for Example 3

27. Primary cultures of bovine chondrocytes were generated as described in Example 1 and plated at 10 million cells in a six-well plate. Following a three day equilibration period, cultures were pretreated for 30 minutes with either 20 μM SC514 or 10 μM SB239063 and their corresponding DMSO controls (0.1% and 0.15%, respectively). IL-1β (10 ng/mL) and IGF-1 (50 ng/mL) were then added to the appropriate wells and incubation continued for another 48 hours. Total RNA was isolated from these cultures using a commercially available RNAqueous-4PCR kit from

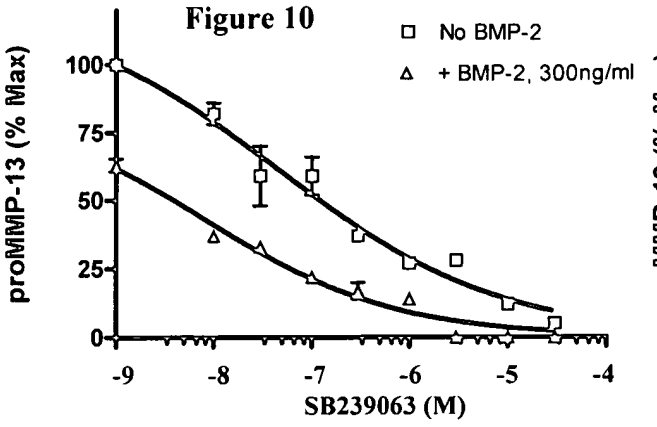
Ambion. RNA was quantified by measuring its OD_{260} and then reverse transcribed into PCR-ready cDNA with a Super Script First-Strand Synthesis kit (Invitrogen) using random hexameres as primers. PCR was then performed using a 3-fold dilution series of each cDNA that corresponded from 5 to 0.06 ng of the input RNA. The MMP-1 primers were designed using DS Gene software (Accelrys) and spanned two introns in order to minimize any problems due to contaminating DNA. The forward primer for MMP-1 was TTGAAAATTACACCCCAGACC and the reverse primer was CGTCCTGAGAAAGCTGAAC. Each PCR reaction also contained 2:8 mixture of 18S RNA primers and 18S competimers (Ambion) to serve as internal standards. PCR conditions were 30 cycles of 94°C denaturation, 56°C primer annealing, and 72°C polymerase extension.

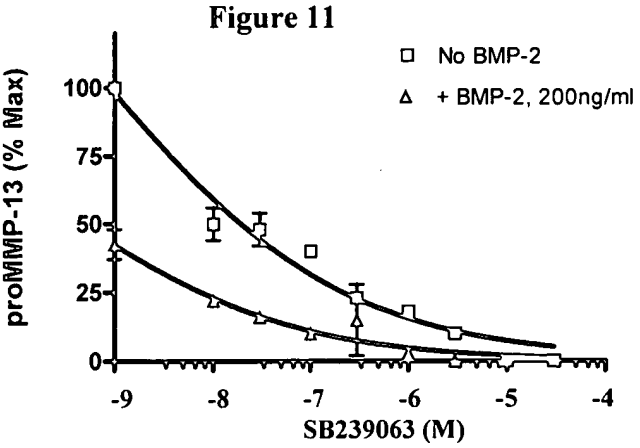
Example 4: The combination of BMP family members and a p38 inhibitor is more effective than each of the respective agents at blocking IL-1β induction of proMMP-13.

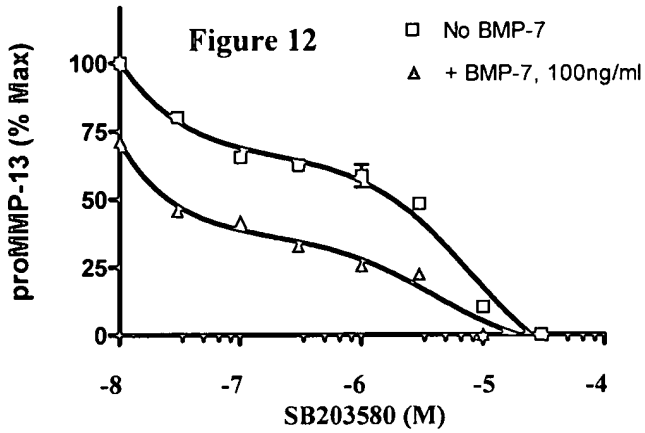
28. MMP-13 is thought to be one of the major proteases involved in the degradation of type II collagen in the osteoarthritic joint. Any agent that blocks its production and/or activity should have a beneficial effect in the osteoarthritic joint [3]. In this example, we show that IL-1β/TNFα induction of proMMP-13 in the C28/I2 human chondrocyte cell line was inhibited in a concentration dependent manner by inhibitors of the p38 MAP kinase (MAPK) signal transduction pathway. In addition, we show that saturating concentrations of Bone Morphogenetic Protein-2 (BMP-2) and BMP-7 partially inhibited proMMP-13 induction, and that the presence of BMP-2 or BMP-7 reduced the amount of p38 MAPK inhibitor that was required for complete inhibition by an order of magnitude.

29. Compilation of data from all studies was complicated by the fact that the degree of inhibition exerted by the BMPs varied from study to study. This variation is possibly explained by the fact that the levels of endogenous BMP antagonists (e.g., noggin) varied from culture to culture. However, despite this variation in the BMP response, the conclusion from each study was the same and multiple representative data sets are shown here.

30. The data shown in **Figure 10** shows results obtained with BMP-2 and the p38 MAPK inhibitor, SB239063. SB239063 induced a concentration-dependent decrease in the IL-1β/TNFα induction of proMMP-13, and virtually complete inhibition was observed at 10 μM and 30 μM SB239063. BMP-2 (300 ng/mL) partially inhibited the induction of proMMP-13 to 68% of the maximal induction. However, in the presence of BMP-2, the amount of SB239063 required to achieve nearly complete inhibition was reduced from 10-30 μM to only 1-3 μM. Similar results were obtained in a second study shown in **Figure 11.** In this study, BMP-2 (200 ng/mL) reduced the induction of proMMP-13 to 43% of the maximal response and nearly complete inhibition (≤15% of maximal response) with the p38 inhibitor occurred at 3-10 μM SB239063. However, in the presence of BMP-2, this level of inhibition was achieved by ten-fold lower concentrations of SB239063 (0.3-1 μM).







The final example shown in **Figure 12** is a single study performed with a second BMP family member, BMP-7, and a second, less specific, p38 inhibitor, SB203580. In this study, BMP-7 (100 ng/mL) had a minimal inhibitory effect on the induction of proMMP-13 to 71% of the maximal induction induced by IL-1β/TNFα. Despite the marginal effect of BMP-7 by itself, it was able to shift the concentration of SB203580 required for significant inhibition (<25% of maximal response) from 10-30 μM to 1-3 μM.

31. Preliminary studies were also performed to determine the effect of other signal transduction inhibitors, either alone or in combination with BMP-2, on the IL-1β/TNFα induction of proMPP13. These inhibitors included a) an ERK1/2 MAPK inhibitor, U0126, b) a JNK MAPK inhibitor, SP600125, and c) a NF-κB inhibitor,

SC514. However, these results were highly variable and additional confirmation is required.

32. In summary, the data presented in Example 4 demonstrate that saturating levels of BMP-2 partially inhibit IL-1β/TNFα induction of proMMP-13 and reduce the amount of p38 inhibitor required for nearly complete inhibition (>85%) by an order of magnitude. Similar results were obtained for a second BMP family member, BMP-7. The clinical development of p38 inhibitors for treatment of inflammatory conditions has been complicated by toxic side effects of these agents on cell types other than the intended target [7]. Therefore, the fact that the combination of BMP-2 or BMP-7 and a p38 inhibitor would require ten-fold lower levels of the p38 inhibitor for maximal inhibition of proMMP-13 production by the chondrocytes in the osteoarthritic joint demonstrates the superiority of the combination compared to the each agent alone, particularly when this combination is delivered locally to further reduce the potential for toxic side effects.

Methods for Example 4

33. The methods for evaluating proMMP-13 production by C28/I2 cells are essentially the same as those described in Example 1. Following the 24-hour incubation in serum-free medium, appropriate cultures were pretreated for 30 minutes with either p38 inhibitor, SB239063 or SB203580, over a concentration range of 0.01μM to 30 μM. At this time, hIL-1β (0.05 ng/mL) and hTNFα (0.5 ng/mL) and/or BMP-2 or -7 (100-300 ng/mL) were added to appropriate cultures and incubation continued for an additional 24 hours. Media samples were collected and frozen until time of assay. The ELISA assay for proMMP-13 was performed as described in Example 1. All cytokines and growth factors were purchased from R & D and the p38 inhibitors from Calbiochem.

34. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

11/06/06
Date

Name: Emma Elizabeth (Betsy) Moore, Ph.D.

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